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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(54) Title:</b> COMPOSITIONS AND METHODS FOR TREATMENT OF MITOCHONDRIAL DISEASES<br><br><b>(57) Abstract</b><br><br>Compounds, compositions, and methods are provided for treatment of disorders related to mitochondrial dysfunction. The methods comprise administering to a mammal a composition containing pyrimidien nucleotide precursors in amounts sufficient to treat symptoms resulting from mitochondrial respiratory chain deficiencies.   |           |   |

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## **COMPOSITIONS AND METHODS FOR TREATMENT OF MITOCHONDRIAL DISEASES**

### **Field of the Invention**

This invention relates generally to compounds and methods for treatment and prevention of diseases, developmental delays, and symptoms related to mitochondrial dysfunction. Pyrimidine nucleotide precursors are administered to a mammal, including a human, for the purpose of compensating for mitochondrial dysfunction and for improving mitochondrial functions.

### **Background of the Invention**

Mitochondria are cellular organelles present in most eukaryotic cells. One of their primary functions is oxidative phosphorylation, a process through which energy derived from metabolism of fuels like glucose or fatty acids is converted to ATP, which is then used to drive various energy-requiring biosynthetic reactions and other metabolic activities. Mitochondria have their own genomes, separate from nuclear DNA, comprising rings of DNA with about 16,000 base pairs in human cells. Each mitochondrion may have multiple copies of its genome, and individual cells may have hundreds of mitochondria.

Mitochondrial dysfunction contributes to various disease states. Some mitochondrial diseases are due to mutations or deletions in the mitochondrial genome. Mitochondria divide and proliferate with a faster turnover rate than their host cells, and their replication is under control of the nuclear genome. If a threshold proportion of mitochondria in a cell is defective, and if a threshold proportion of such cells within a tissue have defective mitochondria, symptoms of tissue or organ dysfunction can result. Practically any tissue can be affected, and a large variety of symptoms may be present, depending on the extent to which different tissues are involved.

A fertilized ovum might contain both normal and genetically defective mitochondria. The segregation of defective mitochondria into different tissues during division of this ovum is a stochastic process, as will be the ratio of defective to normal mitochondria within a given tissue or cell (although there can be positive or negative selection for defective mitochondrial genomes during mitochondrial turnover within cells). Thus, a variety of different pathologic phenotypes can emerge out of a particular point mutation in mitochondrial DNA. Conversely, similar phenotypes can emerge from mutations or deletions affecting different genes within mitochondrial DNA. Clinical symptoms in congenital mitochondrial diseases often manifest in postmitotic tissues with high energy demands like brain, muscle, optic nerve, and myocardium, but other tissues including endocrine glands, liver, gastrointestinal tract, kidney, and hematopoietic tissue are also involved, again depending in part on the segregation of mitochondria during development, and on the dynamics of mitochondrial turnover over time.

In addition to congenital disorders involving inherited defective mitochondria, acquired mitochondrial dysfunction contributes to diseases, particularly neurodegenerative disorders associated with aging like Parkinson's, Alzheimer's, Huntington's Diseases. The incidence of somatic mutations in mitochondrial DNA rises exponentially with age; diminished respiratory chain activity is found universally in aging people. Mitochondrial dysfunction is also implicated in excitotoxic neuronal injury, such as that associated with seizures or ischemia.

Treatment of diseases involving mitochondrial dysfunction has heretofore involved administration of vitamins and cofactors used by particular elements of the mitochondrial respiratory chain. Coenzyme Q (ubiquinone), nicotinamide, riboflavin, carnitine, biotin, and lipoic acid are used in patients with mitochondrial disease, with occasional benefit, especially in disorders directly stemming from primary deficiencies of one of these cofactors. However, while useful in isolated cases, no such metabolic cofactors or vitamins have been shown to have general utility in clinical practice in treating mitochondrial diseases. Similarly, dichloroacetic acid (DCA) has been used to treat mitochondrial cytopathies such as MELAS; DCA inhibits lactate formation and is primarily useful in cases of mitochondrial diseases where excessive lactate accumulation itself is contributing to symptoms. However, DCA does not address symptoms related to mitochondrial insufficiency per se and can be toxic to some patients, depending on the underlying molecular defects.

Mitochondrial diseases comprise disorders caused by a huge variety of molecular lesions or defects, with the phenotypic expression of disease further complicated by stochastic distributions of defective mitochondria in different tissues.

Commonly owned United States Patent 5,583,117 discloses acylated derivatives of cytidine and uridine. Commonly owned application PCT/US 96/10067 discloses the use of acylated pyrimidine nucleosides to reduce the toxicity of chemotherapeutic and antiviral pyrimidine nucleoside analogs.

### **Objects of the Invention**

It is an object of the invention to provide compositions and methods for treating disorders or pathophysiological consequences associated with mitochondrial dysfunction or mitochondrial respiratory chain dysfunction in a mammal, including a human.

It is an object of the invention to provide compounds and compositions that improve tissue resistance to mitochondrial dysfunction in vivo.

It is an object of the invention to provide compositions and methods for treatment of mitochondrial diseases.

It is an object of the invention to provide agents which compensate broadly for mitochondrial deficits involving a wide variety of molecular pathologies, since, in many cases, precise diagnosis of molecular lesions in mitochondrial disorders is difficult.

It is an object of the invention to provide a practical treatment for mitochondrial diseases that is beneficial in the case of mitochondrial electron transport chain deficits regardless of the specific molecular defects.

It is an object of the invention to provide not only for the relatively rare congenital diseases related to mitochondrial DNA defects, but also for significant neuromuscular and

neurodevelopmental disorders that appear in childhood and for common age-related degenerative diseases like Alzheimer's or Parkinson's Diseases.

It is an object of the invention to provide compositions and methods for treatment and prevention of neurodegenerative and neuromuscular disorders.

It is an object of the invention to provide compositions and methods for treatment and prevention of excitotoxic injury to neural tissue.

It is an object of the invention to provide compositions and methods for treatment and prevention of epilepsy.

It is an object of the invention to provide compositions and methods for treatment and prevention of migraine.

It is an object of the invention to provide compositions and methods for preventing death or dysfunction of postmitotic cells in a mammal, including a human.

It is an object of the invention to provide compositions and methods for treatment of neurodevelopmental delay disorders

It is a further object of the invention to provide a composition for treatment or prevention of tissue damage due to hypoxia or ischemia .

It is a further object of this invention to provide compositions and methods for treating or preventing ovarian dysfunction, menopause, or secondary consequences of menopause.

It is a further object of the invention to provide compositions and methods for reducing side effects of cancer chemotherapies due to chemotherapy-induced mitochondrial injury.

It is a further object of the invention to provide a method for diagnosing mitochondrial disease and dysfunction.

## Summary of the Invention

The subject invention provides a method for treating pathophysiological consequences of mitochondrial respiratory chain deficiency in a mammal comprising administering to such a mammal in need of such treatment an amount of a pyrimidine nucleotide precursor effective in reducing the pathophysiological consequences. Additionally, the invention provides a method of preventing pathophysiological consequences of mitochondrial respiratory chain deficiency comprising administering to a mammal an amount of a pyrimidine nucleotide precursor effective in preventing the pathophysiological consequences.

In mitochondrial disease the compounds and compositions of the invention are useful for attenuating clinical sequelae stemming from respiratory chain deficiencies. Respiratory chain deficiencies underlying mitochondrial disease are caused by various factors including congenital or inherited mutations and deletions in mitochondrial DNA, deficits in nuclear-encoded proteins affecting respiratory chain activity, as well as somatic mutations, elevated intracellular calcium, excitotoxicity, nitric oxide, hypoxia and axonal transport defects.

The subject invention provides compounds, compositions, and methods for preventing or reducing death and dysfunction of postmitotic cells bearing mitochondrial respiratory chain deficits.

The subject invention furthermore provides compounds, compositions, and methods for treating neurodevelopmental delays in language, motor, executive function, cognitive, and neuropsychological social skills.

The subject invention also relates to treatment of disorders and conditions that are herein disclosed as conditions to which mitochondrial defects contribute and which therefore are subject to treatment with compounds, and compositions of the invention. These include side effects of cancer chemotherapy like peripheral neuropathies, nephropathies, fatigue, and early menopause, as well as ovulatory abnormalities and normal menopause itself.



The subject invention also relates to a method for diagnosing mitochondrial diseases by treating patients with a pyrimidine nucleotide precursor and assessing clinical benefit in - selected signs and symptoms.

The invention, as well as other objects, features and advantages thereof will be understood more clearly and fully from the following detailed description, when read with reference to the accompanying results of the experiments discussed in the examples below.

### **Detailed Description of the Invention**

The subject invention is related to compounds, compositions, and methods for treating or preventing a variety of clinical disorders secondary to mitochondrial dysfunction, especially deficits in the activity of components of the mitochondrial respiratory chain. Such disorders include congenital mitochondrial cytopathies, neurodevelopmental delays, age-related neurodegenerative diseases, as well as particular diseases affecting the heart, peripheral and autonomic nerves, skeletal muscle, pancreas and other tissues and organs.

#### **A. Definitions**

"Mitochondrial disease" refers to disorders to which deficits in mitochondrial respiratory chain activity contribute in the development of pathophysiology of such disorders in a mammal. This category includes 1) congenital genetic deficiencies in activity of one or more components of the mitochondrial respiratory chain; 2) acquired deficiencies in the activity of one or more components of the mitochondrial respiratory chain, wherein such deficiencies are caused by, inter alia, a) oxidative damage during aging; b) elevated intracellular calcium; c) exposure of affected cells to nitric oxide; d) hypoxia or ischemia; e) microtubule-associated deficits in axonal transport of mitochondria, or f) expression of mitochondrial uncoupling proteins.

The mitochondrial respiratory chain (also known as the electron transport chain) comprises 5 major complexes:

Complex I    NADH:ubiquinone reductase  
Complex II   Succinate:ubiquinone reductase  
Complex III   ubiquinol:cytochrome-c reductase  
Complex IV   cytochrome-c oxidase  
Complex V    ATP synthase

Complexes I and II accomplish the transfer of electrons from metabolic fuels like glycolysis products and fatty acids to ubiquinone (Coenzyme Q), converting it to ubiquinol. Ubiquinol is converted back to ubiquinone by transfer of electrons to cytochrome c in Complex III. Cytochrome c is reoxidized at Complex IV by transfer of electrons to molecular oxygen, - producing water. Complex V utilizes potential energy from the proton gradient produced across the mitochondrial membrane by these electron transfers, converting ADP into ATP, which then provides energy to metabolic reactions in the cell.

Dihydro-orotate dehydrogenase (DHODH), is an enzyme involved in de novo synthesis of uridine nucleotides. DHODH activity is coupled to the respiratory chain via transfer of electrons from dihydro-orotate to ubiquinone; these electrons are then passed onto cytochrome c and oxygen via Complexes III and IV respectively. Only Complexes III and IV are directly involved in pyrimidine biosynthesis. Orotate produced by the action of DHODH is converted to uridine monophosphate by phosphoribosylation and decarboxylation.

"Pyrimidine nucleotide precursors" in the context of the invention are intermediates in either the de novo or salvage pathways of pyrimidine nucleotide synthesis that enter into - pyrimidine synthesis either distal to DHODH (e.g. orotate) or which do not require DHODH activity for conversion to pyrimidine nucleotides (e.g. cytidine, uridine, or acyl derivatives of cytidine or uridine). Also included within the scope of the invention are pyrimidine nucleoside phosphates (e.g. nucleotides, cytidine diphosphocholine, uridine diphosphoglucose); these compounds are degraded to the level of uridine or cytidine prior to entry into cells and anabolism. Acyl derivatives of cytidine and uridine have better oral bioavailability than the parent nucleosides or nucleotides. Orotic acid and esters thereof are converted to uridine nucleotides and are also useful for accomplishing the goals of the invention.

### B. Compounds of the Invention

A primary feature of the present invention is the unexpected discovery that administration of pyrimidine nucleotide precursors is effective in treatment of a large variety of symptoms and disease states related to mitochondrial dysfunction.

Tissue pyrimidine nucleotide levels are increased by administration of any of several precursors. Uridine and cytidine are incorporated into cellular nucleotide pools by phosphorylation at the 5' position; cytidine and uridine nucleotides are interconvertible through enzymatic amination and de-amination reactions. Orotic acid is a key intermediate in de novo biosynthesis of pyrimidine nucleotides. Incorporation of orotic acid into nucleotide pools requires cellular phosphoribosyl pyrophosphate (PRPP). Alternatively (or in addition to provision of exogenous nucleotide precursors), availability of uridine to tissues is increased by administration of compounds which inhibit uridine phosphorylase, the first enzyme in the pathway for degradation of uridine. The compounds of the invention useful in treating mitochondrial diseases and related disorders include uridine, cytidine, orotate, orally bioavailable acyl derivatives or esters of these pyrimidine nucleotide precursors, and inhibitors of the enzyme uridine phosphorylase.

In reference to acyl derivatives of cytidine and uridine, the following definitions pertain:

The term "acyl derivative" as used herein means a derivative of a pyrimidine nucleoside in which a substantially nontoxic organic acyl sub-stituent derived from a carboxylic acid is attached to one or more of the free hydroxyl groups of the ribose moiety of the oxy-purine nucleoside with an ester linkage and/or where such a substituent is attached to the amine substituent on the purine ring of cytidine, with an amide linkage. Such acyl substituents are derived from carboxylic acids which include, but are not limited to, compounds selected from the group consisting of a fatty acid, an amino acid, nicotinic acid, di-carboxylic acids, lactic acid, p-aminobenzoic acid and orotic acid. Advantageous acyl substituents are compounds which are normally present in the body, either as dietary constituents or as intermediary metabolites.

The term "pharmaceutically acceptable salts" as used herein means salts with pharmaceutically acceptable acid or base addition salts of the derivatives, which include, but are not limited to, sulfuric, hydrochloric, or phosphoric acids, or, in the case of orotate, sodium or calcium hydroxides, and cationic amino acids, especially lysine.

The term "amino acids" as used herein includes, but is not limited to, glycine, the L forms of alanine, valine, leucine, isoleucine, phenyl-alanine, tyrosine, proline, hydroxyproline, -serine, threonine, cysteine, cystine, methionine, tryptophan, aspartic acid, glutamic acid, arginine, lysine, histidine, ornithine, hydroxylysine, carnitine, and other naturally occurring amino acids.

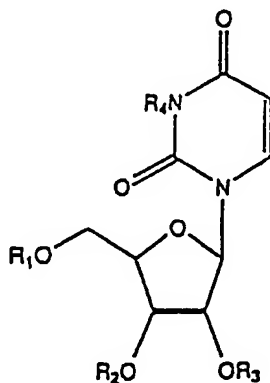
The term "fatty acids" as used herein means aliphatic carboxylic acids having 2-22 carbon atoms. Such fatty acids may be saturated, partially saturated or polyunsaturated.

The term "dicarboxylic acids" as used herein means fatty acids with a second carboxylic acid substituent.

Compounds of the invention have the following structures:

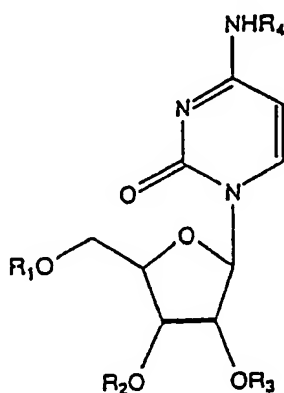
In all cases except where indicated, letters and letters with subscripts symbolizing variable substituents in the chemical structures of the compounds of the invention are applicable only to the structure immediately preceding the description of the symbol.

(1) An acyl derivative of uridine having the formula:



wherein R1, R2, R3 and R4 are the same or different and each is hydrogen or an acyl radical of a metabolite, provided that at least one of said R substituents is not hydrogen, or a pharmaceutically acceptable salt thereof.

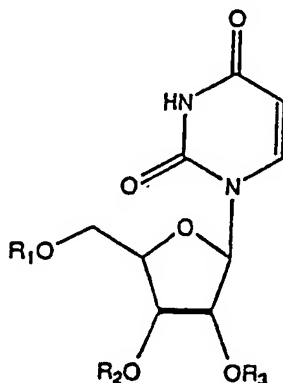
(2) An acyl derivative of cytidine having the formula:



wherein R1, R2, R3 and R4 are the same or different and each is hydrogen or an acyl radical of a metabolite, provided that at least one of said R substituents is not hydrogen, or a pharmaceutically acceptable salt thereof.

The compounds of the invention useful in treating mitochondrial diseases include:

(3) An acyl derivative of uridine having the formula:

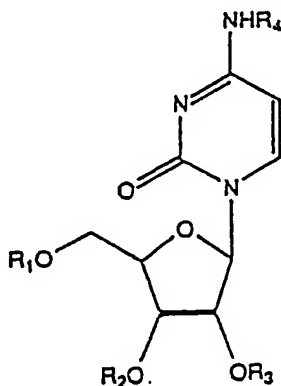


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wherein R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> are the same, or different, and each is hydrogen or an acyl radical of

- a. an unbranched fatty acid with 2 to 22 carbon atoms,
- b. an amino acid selected from the group consisting of glycine, the L forms of alanine, valine, leucine, isoleucine, tyrosine, proline, hydroxyproline, serine, threonine, cystine, cysteine, aspartic acid, glutamic acid, arginine, lysine, histidine, carnitine and ornithine,
- c. a dicarboxylic acid having 3-22 carbon atoms,
- d. a carboxylic acid selected from one or more of the group consisting of glycolic acid, pyruvic acid, lactic acid, enolpyruvic acid, lipoic acid, pantothenic acid, acetoacetic acid, p-aminobenzoic acid, beta-hydroxybutyric acid, orotic acid, and creatine.

(4) An acyl derivatives of cytidine having the formula:

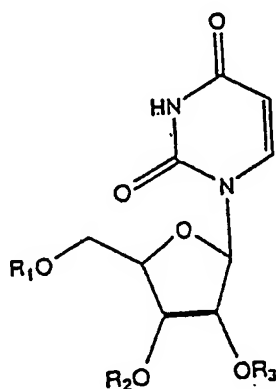


wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> are the same, or different, and each is hydrogen or an acyl radical of

- a. an unbranched fatty acid with 2 to 22 carbon atoms,
- b. an amino acid selected from the group consisting of glycine, the L forms of phenylalanine, alanine, valine, leucine, isoleucine, tyrosine, proline, hydroxyproline, serine, threonine, cystine, cysteine, aspartic acid, glutamic acid, arginine, lysine, histidine, carnitine and ornithine,
- c. a dicarboxylic acid having 3-22 carbon atoms,

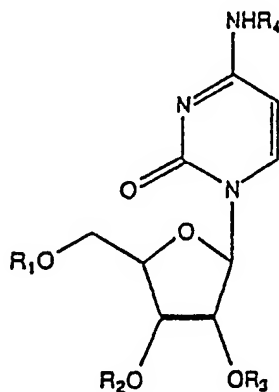
d. a carboxylic acid selected from one or more of the group consisting of glycolic acid, pyruvic acid, lactic acid, enolpyruvic acid, lipoic acid, pantothenic acid, acetoacetic acid, p-aminobenzoic acid, betahydroxybutyric acid, orotic acid, and creatine.

(5) An acyl derivative of uridine having the formula:



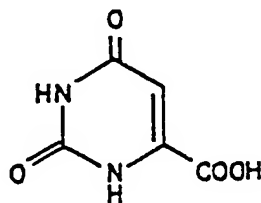
wherein at least one of R1, R2, or R3 is a hydrocarbyloxycarbonyl moiety containing 2-26 carbon atoms and the remaining R substituents are independently a hydrocarbyloxycarbonyl or hydrocarbylcarbonyl moiety or H or phosphate.

(6) An acyl derivative of cytidine having the formula:



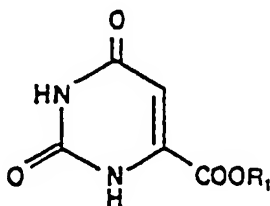
wherein at least one of R1, R2, R3 or R4 is a hydrocarbyloxycarbonyl moiety containing 2-26 carbon atoms and the remaining R substituents are independently a hydrocarbyloxycarbonyl or hydrocarbylcarbonyl moiety or H or phosphate.

(7) Orotic acid or salts thereof:



Pharmaceutically-acceptable salts of orotic acid include those in which the cationic component of the salt is sodium, potassium, a basic amino acid such as arginine or lysine, methylglucamine, choline, or any other substantially nontoxic water soluble cation with a molecular weight less than about 1000 daltons.

8) Alcohol-substituted orotate derivatives:





wherein R1 is a radical of an alcohol containing 1 to 20 carbon atoms joined to orotate via an ester linkage.

Also encompassed by the invention are the pharmaceutically acceptable salts of the above-noted compounds.

Advantageous compounds of the invention are short-chain (2 to 6 carbon atoms) fatty acid esters of uridine or cytidine. Particularly advantageous compounds are triacetyluridine or triacetylcytidine. Such compounds have better oral bioavailability than the parent nucleosides, and are rapidly deacetylated following absorption after oral administration.

Pyruvic acid is useful for treatment of cells with defective mitochondrial function. Cells with reduced capability for mitochondrial oxidative phosphorylation must rely on glycolysis for generation of ATP. Glycolysis is regulated by the redox state of cells. Specifically, NAD<sup>+</sup> is required for optimal glucose flux, producing NADH in the process. In order to maximize energy production from glycolysis, NADH must be reoxidized to NAD<sup>+</sup>. Exogenous pyruvate can reoxidize NADH, in part via a plasma membrane enzyme, NADH Oxidase.

Uridine tripyruvate (2',3',5'-tri-O-pyruvyluridine) provides the benefits of both pyrimidines and pyruvate, delivering both with a single chemical entity, and avoiding the load of sodium, calcium, or other cations in the corresponding salts of pyruvic acid.

#### Inhibitors of uridine phosphorylase

An alternative or complementary strategy for treating mitochondrial diseases involves inhibition of uridine catabolism with an inhibitor of the enzyme uridine phosphorylase.

Examples of inhibitors of uridine phosphorylase that are useful for treatment of mitochondrial disease include but are not limited to 5-benzyl barbiturate or 5-benzylidene barbiturate derivatives including 5-benzyl barbiturate, 5-benzyloxybenzylbarbiturate, 5-benzyloxybenzyl--1-[(1-hydroxy-2--ethoxy)methyl]barbiturate, 5-benzyloxybenzylacetyl--1-[(1-hydroxy-2-ethoxy)methyl] barbiturate, and 5-methoxybenzylacetyl-acyclobarbiturate, 2,2'-

anhydro--5-ethyluridine, 5-ethyl-2-deoxyuridine and acyclocouridine compounds, particularly 5-benzyl substituted acyclocouridine congeners including but not limited to benzylacyclocouridine, benzyloxy-benzyl-acyclo-uridine, aminomethyl--benzyl-acyclocouridine, aminomethyl--benzyloxy-benzylacyclocouridine, hydroxymethyl--benzylacyclocouridine, and hydroxymethyl-benzyloxy-benzyl-acyclocouridine. See also WO 89/09603 and WO 91/16315, hereby incorporated by reference.

### C. Compositions of the Invention

In one embodiment of the invention, novel pharmaceutical compositions comprise as an active agent one or more pyrimidine nucleotide precursors selected from the group consisting of uridine, cytidine, orotic acid or its salts or esters, and acyl derivatives of these pyrimidine nucleotide precursors, together with a pharmaceutically acceptable carrier.

The compositions, depending on the intended use and route of administration, are manufactured in the form of a liquid, a suspension, sprinkles, microcapsules, a tablet, a capsule, a dragee, an injectable solution, or a suppository (see discussion of formulation below).

In another embodiment of the invention, the composition comprises at least one pyrimidine nucleotide precursor and an agent which inhibits the degradation of uridine, such as an inhibitor of the enzyme uridine phosphorylase. Examples of inhibitors of uridine phosphorylase include but are not limited to 5-benzyl barbiturate or 5-benzylidene barbiturate derivatives including 5-benzyl barbiturate, 5-benzyloxybenzyl barbiturate, 5-benzyloxybenzyl-1-[(1-hydroxy-2-ethoxy)methyl] barbiturate, 5-benzyloxybenzylacetyl--1-[(1-hydroxy--2-ethoxy)methyl]barbiturate, and 5-methoxybenzylacetyl-acyclobarbiturate, 2,2'-anhydro-5-ethyluridine, and acyclocouridine compounds, particularly 5-benzyl substituted acyclocouridine congeners including but not limited to benzylacyclocouridine, benzyloxy-benzyl-acyclo-uridine, aminomethyl--benzyl-acyclocouridine, aminomethyl-benzyloxy-benzyl-acyclocouridine, hydroxymethyl--benzylacyclocouridine, and hydroxymethyl-benzyloxy-benzyl-acyclocouridine. Furthermore, it is within the scope of the invention to utilize an inhibitor of uridine phosphorylase alone, without coadministration of a pyrimidine nucleotide precursor, for the

purpose of treating mitochondrial diseases or pathophysiologies associated with mitochondrial respiratory chain dysfunction.

Further embodiments of the invention comprise a pyrimidine nucleotide precursor combined with one or more other agents with protective or supportive activity relative to mitochondrial structure and function. Such agents, presented with recommended daily doses in mitochondrial diseases include, but are not limited to, pyruvate (1 to 10 grams/day), Coenzyme Q (1 to 4mg/kg/day), alanine (1-10 grams/day), lipoic acid (1 to 10mg/kg/day), carnitine (10 to 100 mg/kg/day), riboflavin (20 to 100 mg/day), biotin (1 to 10 mg/day), nicotinamide (20 to 100-mg/day), niacin (20 to 100 mg/day), Vitamin C (100 to 1000mg/day), Vitamin E (200-400 mg/day), and dichloroacetic acid or its salts. In the case of pyruvate, this active agent can be administered as pyruvic acid, pharmaceutically acceptable salts thereof, or pyruvic acid esters having an alcohol moiety containing 2 to 10 carbon atoms.

#### D. Therapeutic Uses of the Compounds and Compositions of the Invention

Diseases related to mitochondrial respiratory chain dysfunction can be divided into several categories based on the origin of mitochondrial defects.

Congenital mitochondrial diseases are those related to hereditary mutations, deletions, or other defects in mitochondrial DNA or in nuclear genes regulating mitochondrial DNA integrity, or in nuclear genes encoding proteins that are critical for mitochondrial respiratory chain function.

Acquired mitochondrial defects comprise primarily 1) damage to mitochondrial DNA due to oxidative processes or aging; 2) mitochondrial dysfunction due to excessive intracellular and intramitochondrial calcium accumulation; 3) inhibition of respiratory chain complexes with endogenous or exogenous respiratory chain inhibitors; 4) acute or chronic oxygen deficiency; and 5) impaired nuclear-mitochondrial interactions, e.g. impaired shuttling of mitochondria in long axons due to microtubule defects, and 6) expression of mitochondrial uncoupling proteins in response to lipids, oxidative damage or inflammation.

The most fundamental mechanisms involved in acquired mitochondrial defects, and which underlie pathogenesis of a variety of forms of organ and tissue dysfunction, include:

**Calcium accumulation:** A fundamental mechanism of cell injury, especially in excitable tissues, involves excessive calcium entry into cells, as a result of either leakage through the plasma membrane or defects in intracellular calcium handling mechanisms. Mitochondria are major sites of calcium sequestration, and preferentially utilize energy from the respiratory chain for taking up calcium rather than for ATP synthesis, which results in a downward spiral of mitochondrial failure, since calcium uptake into mitochondria results in diminished capabilities for energy transduction.

**Excitotoxicity:** Excessive stimulation of neurons with excitatory amino acids is a common mechanism of cell death or injury in the central nervous system. Activation of glutamate receptors, especially of the subtype designated NMDA receptors, results in mitochondrial dysfunction, in part through elevation of intracellular calcium during excitotoxic stimulation. - Conversely, deficits in mitochondrial respiration and oxidative phosphorylation sensitizes cells to excitotoxic stimuli, resulting in cell death or injury during exposure to levels of excitotoxic neurotransmitters or toxins that would be innocuous to normal cells.

**Nitric oxide exposure:** Nitric oxide (~1 micromolar) inhibits cytochrome oxidase (Complex IV) and thereby inhibits mitochondrial respiration (Brown GC, Mol. Cell. Biochem.174:189-192, 1997); moreover, prolonged exposure to NO irreversibly reduces Complex I activity. Physiological or pathophysiological concentrations of NO thereby inhibit pyrimidine biosynthesis. Nitric oxide is implicated in a variety of neurodegenerative disorders including inflammatory and autoimmune diseases of the central nervous system, and is involved in mediation of excitotoxic and post-hypoxic damage to neurons.

**Hypoxia:** Oxygen is the terminal electron acceptor in the respiratory chain. Oxygen deficiency impairs electron transport chain activity, resulting in diminished pyrimidine synthesis as well as diminished ATP synthesis via oxidative phosphorylation. Human cells proliferate and retain viability under virtually anaerobic conditions if provided with uridine and pyruvate (or a - similarly effective agent for oxidizing NADH to optimize glycolytic ATP production).

**Nuclear-mitochondrial interactions:** Transcription of mitochondrial DNA encoding respiratory chain components requires nuclear factors. In neuronal axons, mitochondria must shuttle back and forth to the nucleus in order to maintain respiratory chain activity. If axonal transport is impaired by hypoxia or by drugs like taxol which affect microtubule stability, mitochondria distant from the nucleus undergo loss of cytochrome oxidase activity.

**Mitochondrial Uncoupling Proteins:** Mitochondria are the primary source of free radicals and reactive oxygen species, due to spillover from the mitochondrial respiratory chain, especially when defects in one or more respiratory chain components impairs orderly transfer of electrons from metabolic intermediates to molecular oxygen. To reduce oxidative damage, cells can compensate by expressing mitochondrial uncoupling proteins (UCP), of which several have been identified. UCP-2 is transcribed in response to oxidative damage, inflammatory cytokines, or excess lipid loads, e.g. fatty liver and steatohepatitis. UCP reduce spillover of reactive oxygen species from mitochondria by discharging proton gradients across the mitochondrial inner membrane, in effect wasting energy produced by metabolism and rendering cells vulnerable to energy stress as a trade-off for reduced oxidative injury.

In the nervous system especially, mitochondrial respiratory chain deficits have two generalizable consequences: 1) Delayed or aberrant development of neuronal circuits within the nervous system; and 2) accelerated degeneration of neurons and neural circuits, either acutely or over a period of years, depending on the severity of the mitochondrial deficits and other-precipitating factors. Analogous patterns of impaired development and accelerated degeneration pertain to non-neural tissues and systems as well.

*Mitochondrial dysfunction and pyrimidine biosynthesis*

Cells with severely damaged mitochondria (including total deletion of mitochondrial DNA, with a consequent shutdown of respiratory chain activity) can survive in culture if provided with two agents which compensate for critical mitochondrial functions: uridine and pyruvate. Uridine is required in vitro because a limiting enzyme for de novo synthesis of uridine nucleotides, dihydro-orotate dehydrogenase (DHODH), is coupled to the mitochondrial

respiratory chain, via ubiquinone as a proximal electron acceptor, cytochrome c as an intermediate, and oxygen as a terminal electron acceptor (Loffler et al., Mol. Cell. Biochem.-174:125-129, 1997). DHODH is required for synthesis of orotate, which is then phosphoribosylated and decarboxylated to produce uridine monophosphate (UMP). All other pyrimidines in cells are derived from UMP. Cells from patients with mitochondrial disease due to defects in mitochondrial DNA require exogenous uridine in order to survive outside of the milieu of the body, wherein pyrimidines, derived from other cells or the diet, and transported via the circulation, are prima facie sufficient to support their viability (Bourgeron, et al. Neuromusc. Disord. 3:605-608, 1993). Significantly, intentional inhibition of DHODH with drugs like Brequinar or Leflunomide results in dose-limiting cytotoxic damage to the hematopoietic system and gastrointestinal mucosa, in contrast to the predominant involvement of postmitotic tissues like the nervous system and muscle in clinical mitochondrial disease.

*Pathophysiological consequences of respiratory chain dysfunction*

Mitochondria are critical for the survival and proper function of almost all types of eukaryotic cells. Mitochondria in virtually any cell type can have congenital or acquired defects that affect their function. Thus, the clinically significant signs and symptoms of mitochondrial defects affecting respiratory chain function are heterogeneous and variable depending on the distribution of defective mitochondria among cells and the severity of their deficits, and upon physiological demands upon the affected cells. Nondividing tissues with high energy requirements, e.g. nervous tissue, skeletal muscle and cardiac muscle are particularly susceptible to mitochondrial respiratory chain dysfunction, but any organ system can be affected.

The diseases and symptoms listed below comprise known pathophysiological consequences of mitochondrial respiratory chain dysfunction and as such are disorders in which the compounds and compositions of the invention have therapeutic utility.

Disease symptoms secondary to mitochondrial dysfunction are generally attributed to 1) spillover of free radicals from the respiratory chain; 2) deficits in ATP synthesis leading to cellular energy failure, or 3) apoptosis triggered by release of mitochondrial signals like

cytochrome c which initiate or mediate apoptosis cascades. An unexpected feature of the instant invention is the observation that pyrimidine nucleotide precursors of the invention have therapeutic activity against a large variety of symptoms in patients with mitochondrial disease, as shown in the Examples. This constitutes an important paradigm shift in the understanding of pathogenesis of diseases involving mitochondrial dysfunction, and in understanding how to treat such disorders.

#### Treatment of congenital mitochondrial cytopathies

##### *Mitochondrial DNA defects*

A number of clinical syndromes have been linked to mutations or deletions in mitochondrial DNA. Mitochondrial DNA is inherited maternally, with virtually all of the mitochondria in the body derived from those provided by the oocyte. If there is a mixture of defective and normal mitochondria in an oocyte, the distribution and segregation of mitochondria is a stochastic process. Thus, mitochondrial diseases are often multisystem disorders, and a particular point mutation in mitochondrial DNA, for example, can result in dissimilar sets of signs and symptoms in different patients. Conversely, mutations in two different genes in mitochondrial DNA can result in similar symptom complexes.

Nonetheless, some consistent symptom patterns have emerged in conjunction with identified mitochondrial DNA defects, and these comprise the classic "mitochondrial diseases", some of which are listed immediately below. Nonetheless, an important aspect of the subject invention is the recognition that the concept of mitochondrial disease and its treatment with compounds and compositions of the invention extends to many other disease conditions which are also disclosed herein.

Some of the classical phenotypes of major mitochondrial diseases associated with mutations or deletions of mitochondrial DNA include:

MELAS: (Mitochondrial Encephalomyopathy Lactic Acidemia, and Stroke-like episodes.

MERRF: Myoclonic Epilepsy with "Ragged Red" (muscle) Fibers

MNGIE: Mitochondrial neurogastrointestinal encephalomyopathy

NARP: Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa

LHON: Leber's Hereditary Optic Neuropathy

Leigh's Syndrome (Subacute Necrotizing Encephalomyopathy)

PEO: Progressive External Ophthalmoplegia

Kearns-Sayres Syndrome (PEO, pigmentary retinopathy, ataxia, and heart-block)

Other common symptoms of mitochondrial diseases which may be present alone or in conjunction with these syndromes include cardiomyopathy, muscle weakness and atrophy, developmental delays(involving motor, language, cognitive or executive function),ataxia, epilepsy, renal tubular acidosis, peripheral neuropathy,optic neuropathy, autonomic neuropathy, neurogenic bowel dysfunction, sensorineural deafness, neurogenic bladder dysfunction, dilating cardiomyopathy, migraine, hepatic failure, lactic acidemia, and diabetes mellitus.

In addition, gene products and tRNA encoded by mitochondrial DNA, many proteins involved in, or affecting, mitochondrial respiration and oxidative phosphorylation are encoded by nuclear DNA. In fact, approximately 3000 proteins, or 20% of all proteins encoded by the nuclear genome, are physically incorporated into, or associated with, mitochondria and mitochondrial functions or biogenesis, although only about 100 are directly involved as structural components of the respiratory chain. Therefore, mitochondrial diseases involve not only gene products of mitochondrial DNA, but also nuclear encoded proteins affecting respiratory chain function and mitochondrial structure.



Metabolic stressors like infections can unmask mitochondrial defects that do not necessarily yield symptoms under normal conditions. Neuromuscular or neurological setbacks during infection are a hallmark of mitochondrial disease. Conversely, mitochondrial respiratory chain dysfunction can render cells vulnerable to stressors that would otherwise be innocuous.

Diagnosis of congenital mitochondrial disease is challenging, due to the heterogeneity of symptoms, even between patients affected with the same molecular defect. Deficits in cell and tissue function due to mitochondrial dysfunction can mimic tissue dysfunction caused by problems that do not directly involve mitochondrial defects. Several clinically useful and practical schemes for diagnosis of mitochondrial diseases are known in the art; they typically involve several major criteria (e.g. classical clinical phenotypes like MELAS, NARP or Leigh's Syndrome, extreme (>80%) depressions of respiratory chain complex activity in fresh tissue samples) with a good degree of certainty in establishing the role of respiratory chain dysfunction in disease pathogenesis, and a larger number of minor criteria (e.g. moderate biochemical abnormalities characteristic of respiratory chain defects, symptoms characteristic of mitochondrial diseases without full presentation of one of the classical phenotypes listed above) which individually are less compelling than single major criteria, but which cumulatively provide strong evidence for the contribution of respiratory chain deficits to a particular patient's clinical presentation, as described in Walker et al. (Eur Neurol., 36:260-7, 1996), hereby incorporated by reference.

As is demonstrated in the Examples, compounds and compositions of the invention are useful for treatment of a very broad spectrum of signs and symptoms in mitochondrial diseases with different underlying molecular pathologies. Improvements observed in these and additional patients include but are not limited to reduction of frequency and severity of seizures, migraines, and stroke-like episodes, improvement of weight gain in children with "failure to thrive", amelioration of renal tubular acidosis with concurrent reduction in the need for supplementary bicarbonate, improvement of muscular strength, improvement of speech acquisition, improvement of ataxia, reduction of the frequency and severity of sinus and ear infections, improvement of memory, and amelioration of symptoms of autonomic and peripheral neuropathy. The improvements observed in a broad variety of symptoms which were basically nonresponsive to other forms of metabolic support, e.g. vitamins and cofactors known

to be necessary for proper mitochondrial function (which argues against attribution of benefits to a placebo effect, as does recurrence of symptoms when pyrimidine support is withdrawn) demonstrate a major unexpected insight of the invention, that functional or conditional pyrimidine deficiency underlies a wide variety of dominant symptoms in patients with mitochondrial diseases and that pyrimidine supplementation is sufficient to improve or ameliorate a broad variety of symptoms in such patients. Hitherto, symptoms of mitochondrial disease have been attributed to ATP deficiency, reactive oxygen species generated by the defective respiratory chain, or to cell death triggered by mitochondrial components of the apoptosis cascade. The dose limiting toxicity of inhibitors of *de novo* pyrimidine synthesis are typically due to inhibition of proliferation of rapidly dividing cell types like bone marrow and gut mucosal stem cells. Unexpectedly, therapeutic benefits of compounds and methods of the invention in patients and experimental animals have been demonstrated in tissues comprising nondividing postmitotic cells, e.g. central and peripheral neurons and skeletal and cardiac muscle.

An important feature of the subject invention is the unexpected result that treatment of patients with mitochondrial disease caused by a variety of underlying molecular defects results in clinical improvement in a diverse assortment of symptoms *in vivo* in patients (Examples 1-4). It is significant and further unexpected that clinical benefit has been observed even in patients with normal activity of the two respiratory chain complexes (III and IV) that are directly involved in the electron transfers specifically required for pyrimidine biosynthesis.

Furthermore, it is an unexpected and an important aspect of the invention that higher doses of pyrimidine nucleotide precursors of the invention are typically required for optimal treatment effects in patients with mitochondrial cytopathies than are required for adequate treatment of patients with a virtually complete block in *de novo* pyrimidine synthesis, e.g. homozygotes for Type I orotic aciduria. Optimum doses of a compound of the invention, e.g. triacetyluridine (which is efficiently absorbed after oral administration), for treatment of congenital mitochondrial disease in children are in the range of 1 to 6 grams per m<sup>2</sup> of body surface area (50 to 300 mg/kg, advantageously 100 to 300 mg/kg), whereas total daily *de novo* synthesis of pyrimidines is approximately one gram per day in adults (about 0.5 gram/m<sup>2</sup>).

The broad applicability of the methods of the invention are unexpected and set the compounds and compositions of the invention apart from other therapies of mitochondrial disease that have been attempted e.g. Coenzyme Q, B vitamins, carnitine, and lipoic acid, which generally address very specific reactions and cofactors involved in mitochondrial function and which are therefore useful only in isolated cases. However, such metabolic interventions with antioxidants and cofactors of respiratory chain complexes are compatible with concurrent treatment with compounds and compositions of the invention, and in fact are used to their best advantage in combination with compounds and compositions of the invention.

#### Treatment of neuromuscular degenerative disorders

##### *Friedreich's Ataxia*

A gene defect underlying Friedreich's Ataxia (FA), the most common hereditary ataxia, was recently identified and is designated "frataxin". In FA, after a period of normal development, deficits in coordination develop which progress to paralysis and death, typically between the ages of 30 and 40. The tissues affected most severely are the spinal cord, peripheral nerves, myocardium, and pancreas. Patients typically lose motor control and are confined to wheel chairs, and are commonly afflicted with heart failure and diabetes.

The genetic basis for FA involves GAA trinucleotide repeats in an intron region of the gene encoding frataxin. The presence of these repeats results in reduced transcription and expression of the gene. Frataxin is involved in regulation of mitochondrial iron content. When cellular frataxin content is subnormal, excess iron accumulates in mitochondria, promoting oxidative damage and consequent mitochondrial degeneration and dysfunction.

When intermediate numbers of GAA repeats are present in the frataxin gene intron, the severe clinical phenotype of ataxia may not develop. However, these intermediate-length trinucleotide extensions are found in 25 to 30% of patients with non-insulin dependent diabetes mellitus, compared to about 5% of the nondiabetic population.

Compounds and compositions of the invention are useful for treating patients with disorders related to deficiencies or defects in frataxin, including Friedreich's Ataxia, myocardial dysfunction, diabetes mellitus and complications of diabetes like peripheral neuropathy. Conversely, diagnostic tests for presumed frataxin deficiencies involving PCR tests for GAA intron repeats are useful for identifying patients who will benefit from treatment with compounds and compositions of the invention.

#### *Muscular Dystrophy*

Muscular dystrophy refers to a family of diseases involving deterioration of neuromuscular structure and function, often resulting in atrophy of skeletal muscle and myocardial dysfunction. In the case of Duchenne muscular dystrophy, mutations or deficits in a specific protein, dystrophin, are implicated in its etiology. Mice with their dystrophin genes inactivated display some characteristics of muscular dystrophy, and have an approximately 50% deficit in mitochondrial respiratory chain activity. A final common pathway for neuromuscular degeneration in most cases is calcium-mediated impairment of mitochondrial function. Compounds and compositions of the invention are useful for reducing the rate of decline in muscular functional capacities and for improving muscular functional status in patients with muscular dystrophy.

#### *Multiple sclerosis*

Multiple sclerosis (MS) is a neuromuscular disease characterized by focal inflammatory and autoimmune degeneration of cerebral white matter. Periodic exacerbations or attacks are significantly correlated with upper respiratory tract and other infections, both bacterial and viral, indicating that mitochondrial dysfunction plays a role in MS. Depression of neuronal mitochondrial respiratory chain activity caused by Nitric Oxide (produced by astrocytes and other cells involved in inflammation) is implicated as a molecular mechanism contributing to MS.

Compounds and compositions of the invention are useful for treatment of patients with multiple sclerosis, both prophylactically and during episodes of disease exacerbation.

### Treatment of disorders of neuronal instability

#### *Treatment of seizure disorders*

Epilepsy is often present in patients with mitochondrial cytopathies, involving a range of seizure severity and frequency, e.g. absence, tonic, atonic, myoclonic, and status epilepticus, occurring in isolated episodes or many times daily.

In patients with seizures secondary to mitochondrial dysfunction, compounds and methods of the invention are useful for reducing frequency and severity of seizure activity.

#### *Treatment and prevention of migraine*

Metabolic studies on patients with recurrent migraine headaches indicate that deficits in mitochondrial activity are commonly associated with this disorder, manifesting as impaired - oxidative phosphorylation and excess lactate production. Such deficits are not necessarily due to genetic defects in mitochondrial DNA. Migraineurs are hypersensitive to nitric oxide, an endogenous inhibitor of Cytochrome c Oxidase. In addition, patients with mitochondrial cytopathies, e.g. MELAS, often have recurrent migraines.

In patients with recurrent migraine headaches, compounds, compositions, and methods of the invention are useful for prevention and treatment, especially in the case of headaches refractory to ergot compounds or serotonin receptor antagonists.

As demonstrated in Example 1, compounds and compositions of the invention are useful for treatment of migraines associate with mitochondrial dysfunction.

#### *Treatment of developmental delay*

Delays in neurological or neuropsychological development are often found in children with mitochondrial diseases. Development and remodeling of neural connections requires intensive biosynthetic activity, particularly involving synthesis of neuronal membranes and

myelin, both of which require pyrimidine nucleotides as cofactors. Uridine nucleotides are involved inactivation and transfer of sugars to glycolipids and glycoproteins. Cytidine nucleotides are derived from uridine nucleotides, and are crucial for synthesis of major membrane phospholipid constituents like phosphatidylcholine, which receives its choline moiety from cytidine diphosphocholine. In the case of mitochondrial dysfunction (due to either mitochondrial DNA defects or any of the acquired or conditional deficits like excitotoxic or nitric oxide-mediated mitochondrial dysfunction described above) or other conditions resulting in impaired pyrimidine synthesis, cell proliferation and axonal extension is impaired at crucial stages in development of neuronal interconnections and circuits, resulting in delayed or arrested development of neuropsychological functions like language, motor, social, executive function, and cognitive skills. In autism for example, magnetic resonance spectroscopy measurements of cerebral phosphate compounds indicates that there is global undersynthesis of membranes and membrane precursors indicated by reduced levels of uridine diphospho-sugars, and cytidine nucleotide derivatives involved in membrane synthesis (Minshew et al., Biological Psychiatry 33:762-773, 1993).

Disorders characterized by developmental delay include Rett's Syndrome, pervasive developmental delay (or PDD-NOS: "pervasive developmental delay - not otherwise specified" to distinguish it from specific subcategories like autism), autism, Asperger's Syndrome, and Attention Deficit/Hyperactivity Disorder (ADHD), which is becoming recognized as a delay or lag in development of neural circuitry underlying executive functions.

Compounds and compositions of the invention are useful for treating patients with neurodevelopmental delays involving motor, language, executive function, and cognitive skills. Current treatments for such conditions, e.g. ADHD, involve amphetamine-like stimulants that enhance neurotransmission in some affected underdeveloped circuits, but such agents, which may improve control of disruptive behaviors, do not improve cognitive function, as they do not address underlying deficits in the structure and interconnectedness of the implicated neural circuits.

Compounds and compositions of the invention are also useful in the case of other delays or arrests of neurological and neuropsychological development in the nervous system and somatic development in non-neural tissues like muscle and endocrine glands.

*Treatment of neurodegenerative disorders*

The two most significant severe neurodegenerative diseases associated with aging, Alzheimer's Disease (AD) and Parkinson's Disease (PD), both involve mitochondrial dysfunction in their pathogenesis. Complex I deficiencies in particular are frequently found not only in the nigrostriatal neurons that degenerate in Parkinson's disease, but also in peripheral tissues and cells like muscle and platelets of Parkinson's Disease patients.

In Alzheimer's Disease, mitochondrial respiratory chain activity is often depressed, especially Complex IV (Cytochrome c Oxidase). Moreover, mitochondrial respiratory function altogether is depressed as a consequence of aging, further amplifying the deleterious sequelae of additional molecular lesions affecting respiratory chain function.

Other factors in addition to primary mitochondrial dysfunction underlie neurodegeneration in AD, PD, and related disorders. Excitotoxic stimulation and nitric oxide are implicated in both diseases, factors which both exacerbate mitochondrial respiratory chain deficits and whose deleterious actions are exaggerated on a background of respiratory chain dysfunction.

Huntington's Disease also involves mitochondrial dysfunction in affected brain regions, with cooperative interactions of excitotoxic stimulation and mitochondrial dysfunction contributing to neuronal degeneration. In example 8, a compound of the invention, triacetyluridine, prevents neuronal cell death in a murine model of Huntington's disease.

Compounds and compositions of the invention are useful for treating and attenuating progression of age-related neurodegenerative disease including AD and PD.

*Amyotrophic lateral sclerosis*

One of the major genetic defects in patients with Amyotrophic Lateral Sclerosis (ALS; Lou Gehrig's Disease; progressive degeneration of motor neurons, skeletal muscle atrophy, inevitably leading to paralysis and death) is mutation or deficiency in Copper-Zinc Superoxide Dismutase (SOD1), an antioxidant enzyme. Mitochondria both produce and are primary targets for reactive oxygen species. Inefficient transfer of electrons to oxygen in mitochondria is the most significant physiological source of free radicals in mammalian systems. Deficiencies in antioxidants or antioxidant enzymes can result in or exacerbate mitochondrial degeneration. Mice transgenic for mutated SOD1 develop symptoms and pathology similar to those in human ALS. The development of the disease in these animals has been shown to involve oxidative destruction of mitochondria followed by functional decline of motor neurons and onset of clinical symptoms (Kong and Xu, J. Neurosci. 18:3241-3250, 1998). Skeletal muscle from ALS patients has low mitochondrial Complex I activity (Wiedemann et al., J. Neurol. Sci 156:65-72, 1998).

Compounds, compositions, and methods of the invention are useful for treatment of ALS, for reversing or slowing the progression of clinical symptoms.

*Protection against ischemia and hypoxia*

Oxygen deficiency results in both direct inhibition of mitochondrial respiratory chain activity by depriving cells of a terminal electron acceptor for Cytochrome c reoxidation at Complex IV, and indirectly, especially in the nervous system, via secondary post-anoxic excitotoxicity and nitric oxide formation.

In conditions like cerebral anoxia, angina or sickle cell anemia crises, tissues are relatively hypoxic. In such cases, compounds of the invention provide protection of affected tissues from deleterious effects of hypoxia, attenuate secondary delayed cell death, and accelerate recovery from hypoxic tissue stress and injury.



Compounds and compositions of the invention are useful for preventing delayed cell death (apoptosis in regions like the hippocampus or cortex occurring about 2 to 5 days after an episode of cerebral ischemia) after ischemic or hypoxic insult to the brain.

#### *Renal tubular acidosis*

Acidosis due to renal dysfunction is often observed in patients with mitochondrial disease, whether the underlying respiratory chain dysfunction is congenital or induced by ischemia or cytotoxic agents like cisplatin. Renal tubular acidosis often requires administration of exogenous sodium bicarbonate to maintain blood and tissue pH.

In Example 3, administration of a compound of the invention caused an immediate reversal of renal tubular acidosis in a patient with a severe Complex I deficiency. Compounds and compositions of the invention are useful for treating renal tubular acidosis and other forms of renal dysfunction caused by mitochondrial respiratory chain deficits.

#### *Age-related neurodegeneration and cognitive decline*

During normal aging, there is a progressive decline in mitochondrial respiratory chain function. Beginning about age 40, there is an exponential rise in accumulation of mitochondrial DNA defects in humans, and a concurrent decline in nuclear-regulated elements of mitochondrial respiratory activity.

de Grey (Bioessays, 19:161-167, 1998) discussed mechanisms underlying the observation that many mitochondrial DNA lesions have a selection advantage during mitochondrial turnover, especially in postmitotic cells. The proposed mechanism is that mitochondria with a defective respiratory chain produce less oxidative damage to themselves than do mitochondria with intact functional respiratory chains (mitochondrial respiration is the primary source of free radicals in the body). Therefore, normally-functioning mitochondria accumulate oxidative damage to membrane lipids more rapidly than do defective mitochondria, and are therefore "tagged" for degradation by lysosomes. Since mitochondria within cells have a half life of about 10 days, a selection advantage can result in rapid replacement of functional -

mitochondria with those with diminished respiratory activity, especially in slowly dividing cells. The net result is that once a mutation in a gene for a mitochondrial protein that reduces oxidative damage to mitochondria occurs, such defective mitochondria will rapidly populate the cell, diminishing or eliminating its respiratory capabilities. The accumulation of such cells results in aging or degenerative disease at the organismal level. This is consistent with the progressive mosaic appearance of cells with defective electron transport activity in muscle, with cells almost devoid of Cytochrome c Oxidase (COX) activity interspersed randomly amidst cells with normal activity, and a higher incidence of COX-negative cells in biopsies from older subjects. The organism, during aging, or in a variety of mitochondrial diseases, is thus faced with a situation in which irreplaceable postmitotic cells (e.g. neurons, skeletal and cardiac muscle) must be preserved and their function maintained to a significant degree, in the face of an inexorable progressive decline in mitochondrial respiratory chain function. Neurons with dysfunctional mitochondria become progressively more sensitive to insults like excitotoxic injury. Mitochondrial failure contributes to most degenerative diseases (especially neurodegeneration) that accompany aging.

Congenital mitochondrial diseases often involve early-onset neurodegeneration similar in fundamental mechanism to disorders that occur during aging of people born with normal mitochondria. The demonstration disclosed in the Examples that compounds and compositions of the invention are useful in treatment of congenital or early-onset mitochondrial disease provides direct support for the utility of compounds and compositions of the invention for treatment of age-related tissue degeneration.

Compounds and compositions of the invention are useful for treating or attenuating cognitive decline and other degenerative consequences of aging.

#### *Mitochondria and cancer chemotherapy*

Mitochondrial DNA is typically more vulnerable to damage than is nuclear DNA for several reasons:

1. Mitochondrial DNA has a less sophisticated repair system than does nuclear DNA.
2. Virtually all of the mitochondrial DNA strands encode important proteins, so that any defect will potentially affect mitochondrial function. Nuclear DNA contains long regions that do not encode proteins, wherein mutations or damage are essentially inconsequential.
3. Defective mitochondria often have a selection advantage over normal, active ones during proliferation and turnover.
4. Mitochondrial DNA is not protected by histones

Empirically, mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in cells subjected to oxidative stress or cancer chemotherapy agents like cisplatin due to both greater vulnerability and less efficient repair of mitochondrial DNA. Although mitochondrial DNA may be more sensitive to damage than nuclear DNA, it is relatively resistant, in some situations, to mutagenesis by chemical carcinogens. This is because mitochondria respond to some types of mitochondrial DNA damage by destroying their defective genomes rather than attempting to repair them. This results in global mitochondrial dysfunction for a period after cytotoxic chemotherapy. Clinical use of chemotherapy agents like cisplatin, mitomycin, and cytoxan is often accompanied by debilitating "chemotherapy fatigue", prolonged periods of weakness and exercise intolerance which may persist even after recovery from hematologic and gastrointestinal toxicities of such agents.

Compounds, compositions, and methods of the invention are useful for treatment and prevention of side effects of cancer chemotherapy related to mitochondrial dysfunction. This use of pyrimidine nucleotide precursors for attenuation of cancer chemotherapy side effects is mechanistically and biochemically distinct from toxicity reduction of cytotoxic anticancer - pyrimidine analogs by pyrimidine nucleotide precursors, which is mediated through biochemical competition at the level of nucleotide antimetabolites.

Example 5 illustrates the protective effect of oral triacetylrudine in protecting against taxol-induced neuropathy.

Furthermore, hepatic mitochondrial redox state is one contributor to appetite regulation. Cancer patients often display "early satiety", contributing to anorexia, weight loss, and cachexia. Energy metabolism is often seriously disrupted in cancer patients, with energy-wasting futile cycles of hyperactive tumor glycolysis producing circulating lactate, which is converted by the liver back to glucose. Chemotherapy-induced mitochondrial injury further contributes to metabolic disruption.

As indicated in Example 2, treatment with a compound of the invention resulted in improved appetite in a patient with mitochondrial disease.

#### *Mitochondria and ovarian function*

A crucial function of the ovary is to maintain integrity of the mitochondrial genome in oocytes, since mitochondria passed onto a fetus are all derived from those present in oocytes at the time of conception. Deletions in mitochondrial DNA become detectable around the age of menopause, and are also associated with abnormal menstrual cycles. Since cells cannot directly detect and respond to defects in mitochondrial DNA, but can only detect secondary effects that affect the cytoplasm, like impaired respiration, redox status, or deficits in pyrimidine synthesis, such products of mitochondrial function participate as a signal for oocyte selection and follicular atresia, ultimately triggering menopause when maintenance of mitochondrial genomic fidelity and functional activity can no longer be guaranteed. This is analogous to apoptosis in cells with DNA damage, which undergo an active process of cellular suicide when genomic fidelity can no longer be achieved by repair processes. Women with mitochondrial cytopathies affecting the gonads often undergo premature menopause or display primary cycling abnormalities. Cytotoxic cancer chemotherapy often induces premature menopause, with a consequent increased risk of osteoporosis. Chemotherapy-induced amenorrhea is generally due to primary ovarian failure. The incidence of chemotherapy-induced amenorrhea increases as a function of age in premenopausal women receiving chemotherapy, pointing toward mitochondrial involvement. Inhibitors of mitochondrial respiration or protein synthesis inhibit hormone-induced ovulation, and furthermore inhibit production of ovarian steroid hormones in response to pituitary gonadotropins. Women with

Downs syndrome typically undergo menopause prematurely, and also are subject to early onset of Alzheimer-like dementia. Low activity of cytochrome oxidase is consistently found in tissues of Downs patients and in late-onset Alzheimer's Disease.

Appropriate support of mitochondrial function or compensation for mitochondrial dysfunction therefore is useful for protecting against age-related or chemotherapy-induced menopause or irregularities of menstrual cycling or ovulation. Compounds and compositions of the invention, including also antioxidants and mitochondrial cofactors, are useful for treating and preventing amenorrhea, irregular ovulation, menopause, or secondary consequences of menopause.

In Example 1, treatment with a compound of the invention resulted in shortening of the menstrual cycle. Since the patient was in a persistent luteal phase, her response indicates that the administered pyrimidine nucleotide precursor reversed hyporesponsiveness to pituitary gonadotropins, which were presumably elevated to compensate for the ovarian hyporesponsiveness of mitochondrial origin.

#### *Diagnosis of mitochondrial disease*

The striking response of patients with mitochondrial disease to administration of compounds of the invention indicates that a clinical response to a pyrimidine nucleotide precursor administered according to the methods of the subject invention has diagnostic utility to detect possible mitochondrial disease. Molecular diagnosis of molecular lesions underlying mitochondrial dysfunction is difficult and costly, especially when the defect is not one of the more common mutations or deletions of mitochondrial DNA. Definitive diagnosis of mitochondrial disease often requires muscle biopsies, but even this invasive measure only works if mitochondrial defects are present in muscle. Since the compounds and compositions of the invention are safe when administered in accord with the methods of the subject invention, therapeutic challenge with a pyrimidine nucleotide precursor is an important diagnostic probe for suspected mitochondrial disease, especially when used in conjunction with tests for various other aspects of mitochondrial dysfunction.

For diagnosis of congenital mitochondrial cytopathy, daily doses of 50 to 300 mg/kg of a pyrimidine nucleotide precursor of the invention are administered to a patient for a period of one to twelve weeks and clinical signs and symptoms are monitored for changes. Improvements observed in the patients described in the Examples and additional patients include but are not limited to reduction of frequency and severity of seizures, migraines, and stroke-like episodes, improvement of weight gain in children with "failure to thrive", amelioration of renal tubular acidosis with concurrent reduction in the need for supplementary bicarbonate, improvement of muscular strength, improvement of speech acquisition, improvement of ataxia, improvement of hypotonia, reduction of the frequency and severity of sinus and ear infections, improvement of memory, and amelioration of symptoms of autonomic and peripheral neuropathy. In one embodiment of the invention, other tests of mitochondrial function are also used to provide evidence for diagnosis of mitochondrial disease. Diagnosis typically requires cumulative consideration of a number of corroborative tests with differing degrees of reliability, as described in Walker et al, (Eur Neurol., 36:260-7, 1996). Therapeutic responsiveness to a pyrimidine nucleotide precursor of the invention is primarily useful as an additional minor criterion in this diagnostic scheme, since it is possible that therapeutic benefits may occur after administration of pyrimidine nucleotide precursors that are not mediated solely by compensation for respiratory chain deficits. Since the nature and severity of symptoms of mitochondrial diseases are heterogeneous and variable between patients, efficacy of exogenous pyrimidine nucleotide precursors is typically assessed by selecting dominant symptoms in a patient and monitoring their severity with as quantitative a scale as is feasible during a course of therapy. If a possible placebo effect is suspected, blinded switching of the patient from drug to an appropriate placebo is optionally used in an individual patient. Assessment of clinical benefit can require considerable skill and experience, but such skill is in the province of practitioners of the art of treating patients with multisystem metabolic diseases, and as such does not constitute undue experimentation, in view of the severity of this class of diseases. The examples cited below of clinical treatment of patients with mitochondrial diseases with triacetyluridine, a compound of the invention, exemplify the feasibility of determining clinical benefit in individual patients.

#### E. Administration and Formulation of Compounds and Compositions of the Invention

In the case of all of the specific therapeutic targets for pyrimidine nucleotide precursor therapy of mitochondrial disease, compounds of the invention are typically administered one to three times per day. Acyl derivatives of uridine and cytidine are administered orally in doses of 10 to 500 mg/kg of body weight per day, with variations within this range depending on the amount required for optimal clinical benefit. Generally, optimum doses are between 50 and 300 mg/kg/day (advantageously 100 to 300 mg/kg/day), divided into two or three separate doses taken 6 to 12 hours apart. Uridine and cytidine are less efficiently absorbed than are acyl derivatives of these two nucleosides, so that higher doses are required for therapeutic benefit comparable to that achieved with acyl derivatives. Osmotic diarrhea limits the amount of uridine or cytidine (or other derivatives like cytidine diphosphocholine) that can be administered to a patient, so that in most cases acyl derivatives of cytidine and uridine are more effective than the parent compounds, with fewer side effects. Doses of cytidine and uridine used to accomplish the purposes of the invention range from 50 to 1000 mg/kg/day, advantageously 100 to 1000 mg/kg/day, depending on the balance between therapeutic efficacy and tolerability. Orotate or alcohol esters of orotate are administered orally in doses ranging from 20 to 200 mg/kg/day, again depending on the amount needed to achieve an optimal therapeutic effect in a particular disease state involving mitochondrial respiratory chain dysfunction. The dose of pyrimidine nucleotide precursor of the invention required for a particular disease or patient will also depend in part on the severity of the disease.

In any individual patient with a disease characterized or caused by mitochondrial dysfunction, an effective dose of a pyrimidine nucleotide precursor of the invention is typically determined empirically. In congenital mitochondrial diseases, also known as mitochondrial cytopathies or mitochondrial encephalomyopathies, the clinical presentation of signs and symptoms is generally heterogeneous and variable between patients. Clinical benefit following administration of a compound of the invention is determined by monitoring a set of symptoms and assessing their severity over time, e.g. at monthly intervals. Three to five dominant symptoms are selected for this purpose, and the degree of amelioration judged to constitute clinical benefit is often a matter of clinical judgment. In treatment of patients with complex metabolic disorders, such assessment does not constitute undue burden of experimentation,

especially given the severity (often life threatening) of mitochondrial cytopathies and the costly nature of their care. Compensation for mitochondrial or other metabolic defects as early as possible in the patients life can make a very large difference versus intervention after development of the brain and body achieves stasis after puberty. It is therefore worthwhile for considerable effort to be expended on diagnosis and treatment of complex metabolic diseases, especially in developing children. The examples cited below of clinical improvement following administration of a compound of the invention to patients with mitochondrial diseases demonstrate the feasibility and value of such treatment and assessment.

In the case of most diseases with less heterogeneity in clinical presentation than mitochondrial disease, there exist in the art appropriate validated assessment scales for determining efficacy of drug treatments. Prior to conducting clinical studies to determine the doses of pyrimidine nucleotide precursors of the invention for treatment of the disease conditions disclosed in the instant specification, appropriate doses for individual patients are determined by evaluating clinical response (including brain MRI images and other indices, e.g. biochemical measurements, that may not necessarily be clinically apparent simply by observation of the patient's symptoms) according to quantitative disease assessment scales. In all cases, the dominant symptoms of a particular disease state are monitored over time to determine whether an improvement of signs and symptoms or attenuation of clinical decline occurs, as is common in the art of medicine. Prior to dose determination in blinded clinical studies, the response of a given patient to a pyrimidine nucleotide precursor of the invention is be differentiated from a possible placebo effect simply by blinded switchover from drug to placebo for a period of several weeks.

In the case of patients unable to receive oral medications, compounds of the invention, especially uridine, cytidine, and orotate esters can be administered, as required, by prolonged intravenous infusion, delivering daily doses of 10 to 500 mg/kg/day.

The pharmacologically active compounds optionally are combined with suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds. These are administered as tablets, suspensions, solutions, dragees, capsules, or suppositories. The compositions are administered for example orally,



rectally, vaginally, or released through the buccal pouch of the mouth, and may be applied in solution form by injection, orally or by topical administration. The compositions may contain from about 0.1 to 99 percent, preferably from about 50 to 90 percent of the active compound(s), together with the excipient(s).

For parenteral administration by injection or intravenous infusion, the active compounds are suspended or dissolved in aqueous medium such as sterile water or saline solution. - Injectable solutions or suspensions optionally contain a surfactant agent such as polyoxyethylenesorbitan esters, sorbitan esters, polyoxyethylene ethers, or solubilizing agents like propylene glycol or ethanol. The solution typically contains 0.01 to 5% of the active compounds.

Suitable excipients include fillers such as sugars, for example lactose, sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch or potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethyl cellulose, sodium carboxymethyl cellulose and/or polyvinyl pyrrolidone.

Auxiliaries include flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated sugar solutions are used, which optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetyl cellulose phthalate or hydroxypropylmethyl cellulose phthalate are used. Dyestuffs or pigments are optionally added to the tablets or dragee coatings, for example, for identification or in order to characterize different compound doses.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use

are obtained by combining the active compound(s) with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Other pharmaceutical preparations which are useful for oral delivery include push-fit capsules made of gelatin, as well as soft-sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules contain the active compound(s) in the form of granules which optionally are mixed with fillers such as lactose, binders such as starches and/or lubricants such as talc or magnesium stearate, and, optionally stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids such as fatty oils, liquid paraffin, or polyethylene glycols. In addition, stabilizers optionally are added.

Pharmaceutical preparations which are used rectally include, for example, suppositories which consist of a combination of active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols. In addition, gelatin rectal capsules which consist of a combination of the active compounds with a base are useful. Base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons. In another embodiment of the invention, an enema formulation is used, which optionally contains viscosity-increasing excipients like methylcellulose, hydroxypropylmethylcellulose, carboxymethylcellulose, carbopol, glycerine polyacrylates, or other hydrogels.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water soluble form, for example, water soluble salts.

In addition, suspensions of the active compounds as appropriate in oily injection suspensions are administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions optionally include substances which increase the viscosity of the suspension which include, for example, sodium carboxymethyl cellulose, sorbitol and/or dextran. The suspension optionally contains stabilizers.

#### F. Synthesis of the Compounds of the Invention

Acyl derivatives of cytidine and uridine are synthesized typically by acylation methods involving reaction of acid chlorides or acid anhydrides with cytidine or uridine.

The synthesis of 2',3',5'-tri-O-pyruvyluridine is shown in Example 6.

\* \* \*

The following examples are illustrative, but not limiting of the methods and compositions of the present invention. Other suitable modifications and adaptations of a variety of conditions and parameters normally encountered in clinical therapy which are obvious to those skilled in the art are within the spirit and scope of this invention.

### **Examples**

#### Example 1: Treatment of a multisystem mitochondrial disorder with triacetyluridine

A 29 year old woman with a partial Complex I deficiency, and whose son was diagnosed with mitochondrial disease leading to stroke-like episodes, ataxia, and encephalopathy, presented with a multisystem mitochondrial disorder. Signs and symptoms included hemiplegic/aphasic migraines, grand-mal seizures, neurogenic bowel and bladder dysfunction, requiring catheterization approximately four times per day, dysphagia, autonomic and peripheral polyneuropathy producing painful paresthesias, tachycardia/bradycardia syndrome, and poor functional capacity with inability to climb a flight of stairs without stopping to rest, and declining cognitive performance with episodes of clouded sensorium and poor memory lasting hours to days.

After beginning treatment with 0.05 mg/kg/day of oral triacetyluridine, and for a duration of at least 6 months, this patient has not had seizures or migraines; her paresthesias related to peripheral neuropathy have resolved. She is able to void spontaneously on most days, requiring catheterization only once or twice per week. After 6 weeks of treatment with

triacetyluridine, this patient was able to walk a full mile, which she has been unable to do for the past two years because of inadequate functional capacity. Her episodes of bradycardia during sleep and tachycardia during exertion have reduced infrequency; prior to treatment, tachycardia with a heart rate greater than 140 bpm occurred upon simple rise to stand, and after 6 weeks of triacetyluridine, tachycardia occurred only on hills and stairs. Her sensorium has cleared and memory deficits have improved markedly.

During treatment, this patients' menstrual cycles shortened from 4 weeks to two weeks, and she displayed a persistent luteal phase as evaluated by estradiol, progesterone, FSH and LH measurements. After several months, her cycle normalized to 4 weeks.

This patient demonstrates important features of the subject invention, in that 1) the compound of the invention caused improvements in virtually all features of a complex multisystem disease related to mitochondrial dysfunction in a variety of tissues, and that 2) compounds of the invention are unexpectedly useful for treating disease conditions related to a partial Complex I deficiency, which affects a portion of the mitochondrial respiratory chain that is outside of the sequence of electron transfers directly involved in de novo pyrimidine biosynthesis.

The transient shortening of this patient's menstrual cycle is interpreted as an improvement of ovarian function caused by triacetyl uridine in the face of excessive hormonal stimulation by which the neuroendocrine system was attempting to compensate for ovarian dysfunction. Feedback between the ovaries and the hypothalamus led to gradual normalization of cycle time.

#### Example 2: Treatment of refractory epilepsy

An 11 year old boy had refractory epilepsy since age 4.5, apparently due to a multiple mitochondrial DNA deletion syndrome. In December 1997, his condition deteriorated, including two admissions to an intensive care unit for crescendo epilepsy. Even with aggressive regimens of standard anticonvulsive therapy, this patient was having 8 to 10 grand-mal seizures

per night, leaving him unable to attend school regularly or participate in sports activities. He also developed upper lip automaticity.

In the first three days after beginning treatment with oral triacetyluridine (initially at a dose of 0.05 g/kg/day, and incrementally increased to 0.1 and then 0.24 g/kg/day over the course of several weeks), there were no seizures, and involuntary lip movements ceased. There has subsequently been some recurrence of seizures especially during episodes of infection, though at a much lower frequency than prior to treatment with triacetyluridine. This patient has been able to return to school and resume active participation in sports. His appetite, cognitive function, and fine motor coordination have improved during therapy, resulting in improved academic performance and in outstanding performance in sports activities like baseball.

#### Example 3: Treatment of renal tubular acidosis

A 2 year-old girl, with Leigh's Syndrome (subacute necrotizing encephalopathy) associated with severe Complex I deficiency, displayed renal tubular acidosis requiring intravenous administration of 25 mEq per day of sodium bicarbonate. Within several hours after beginning intragastric treatment with triacetyluridine at 0.1 g/mg/day, her renal tubular acidosis resolved and supplementary bicarbonate was no longer required to normalize blood pH. Triacetyluridine also resulted in rapid normalization of elevated circulating amino acid concentrations, and maintained lactic acid at low levels after withdrawal of dichloroacetate treatment, which was previously required to prevent lactic acidosis.

#### Example 4: Treatment of developmental delay

A 4.5 year-old girl with epilepsy, ataxia, language delay, and fat intolerance, and dicarboxylic aciduria was treated with triacetyluridine at a daily dose of 0.1 to 0.3 g/kg/day. Such treatment resulted in a 50% decline in seizure frequency, improvement of ataxia and motor coordination, restoration of dietary fat tolerance, and rapidly accelerated development of expressive language capabilities.

Example 5: Prevention of taxol-induced neuropathy

Peripheral neuropathy is a frequent, and often dose-limiting, side effect of important anticancer agents like cisplatin and taxol. In the case of taxol, sensory neuropathy occurs several days after administration. Taxol's mechanism of action involves stabilization of microtubules, which is useful for treating cancers, but is deleterious to peripheral neurons. - Microtubule stabilization impairs axonal transport of cellular components. Mitochondria shuttle between the cell body and terminals of neurons, so that the expression of mitochondrial - respiratory chain components can be regulated by nuclear transcription factors. During inhibition of mitochondrial shuttling, mitochondria distant from the nucleus undergo decline in expression of respiratory chain subunits encoded by the mitochondrial genome, due to inadequate exposure to mtDNA transcription factors, resulting in regional neuronal energy failure and other consequences of mitochondrial dysfunction.

Two groups of 10 mice each were treated with taxol, 21.6mg/kg/day for 6 consecutive days by intraperitoneal injection. An additional group of 10 mice received injections of vehicle alone. One of the groups of taxol-treated mice received oral triacetyluridine, 4000 mg/kg b.i.d. Nine days after the initiation of taxol treatments, nociceptive sensory deficits weretested by determining tail-flick latency after exposure of the tip of the tail to focused thermal radiation with an infrared heat lamp. In this system, delays in the tail-flick response to radiant heat correlate with sensory nerve deficits.

| Group:                   | Tail flick latency |
|--------------------------|--------------------|
| Control (no taxol)       | 10.8 ± 0.5 seconds |
| Taxol                    | 16.0 ± 3.1 seconds |
| Taxol + triacetyluridine | 11.9 ± 0.7 seconds |

Taxol treatment impaired responses to painful stimuli as an index of toxic sensory neuropathy. Oral triacetyluridine treatment significantly attenuated taxol-induced alterations in tail-flick latency.

Example 6: Synthesis of Uridine Pyruvate

A. The preparation of pyruvyl chloride was accomplished by the reaction of alpha, alpha-dichloromethyl methyl ether and pyruvic acid using the procedure of Ottenheim and Man (Synthesis, 1975, p. 163).

B. Uridine (3.0 g, 12 mmol) was dried by toluene azeotrope under vacuum (3x), and then dissolved in DMF (20 mL) and pyridine (20 mL). The resultant solution was cooled to -10 degrees C and 6.0 mL of pyruvyl chloride (produced in step A above) was added dropwise. The reaction mixture was stirred at room temperature under argon for 24 hours. Analysis by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) showed the consumption of uridine. The reaction mixture was evaporated to dryness and partitioned between CH<sub>2</sub>Cl<sub>2</sub> and aqueous sodium bicarbonate. The organic layer was washed with water, aqueous HCl (pH 3.0), and water; dried over sodium sulfate; concentrated; and purified using flash chromatography (silicagel, 5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) to yield 1.4 g of uridine pyruvate, or 2',3',5'-tri-O-pyruvyluridine.

Example 7: Therapeutic effect of oral triacetyluridine in the MPTP model of Parkinson's disease (PD) and mitochondrial dysfunction

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a complex I (NADH dehydrogenase) mitochondrial respiratory chain inhibitor that is used to induce dopaminergic cell loss (Varastet *et al.*, Neuroscience, 63: 47-56, 1994). This toxin is currently widely used as an animal model for PD (Bezard *et al.*, Exp Neurol, 148: 288-92, 1997).

Male C57/BL6 mice that were 6-9 months old weighing 30-40g from Taconic Farms were used in the MPTP studies (n=7/group). MPTP (30 mg/kg i.p.) was given b.i.d. for 1.5 days. TAU was administered b.i.d. 4g/kg p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL solution, 2 hours prior to toxin administration and until the day before sacrifice.

Eight days after stopping injection of MPTP, the mice were sacrificed by CO<sub>2</sub> and the striata from both sides were dissected out on cold surface. The striatum was frozen on dry ice. The dopaminergic neuronal survival was assessed by striatal dopamine (DA) content. The dopamine content was assayed by a radioenzymatic method under GLP conditions, but DA can also be measured using high pressure liquid chromatography with electrochemical detection as previously described (Friedemann & Gerhardt, Neurobiol Aging, 13: 325-32, 1992). There was a decreased mortality in the MPTP treated mice due to TAU treatment. The mortality in the control + MPTP mice was 71.4% compared to 28.6% in the TAU + MPTP treatment group. There was also a neuroprotective effect of PN401 treatment on the decrease in DA content due to MPTP.

#### Effect of TAU on MPTP-induced decrease in striatal DA content

| Treatment         | Striatal DA* |
|-------------------|--------------|
| Control + Control | 147 ± 13.0   |
| TAU + Control     | 93.8 ± 10.7  |
| Control + MPTP    | 9.2 ± 2.2    |
| TAU + MPTP        | 37.9 ± 7.4   |

\* Data are represented as ng DA/mg protein (mean ± SEM).

A second study using MPTP (25 mg/kg i.p. b.i.d. for 2 days) was performed. Male C57/BL6 mice that were 6-9 months old weighing 30-40g from Taconic Farms were used in the MPTP studies (n=6/group). MPTP (30 mg/kg i.p.) was given b.i.d. for 2 days. TAU was administered b.i.d. 4g/kg bw p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL solution 2 hours prior to toxin administration and until the day before sacrifice. TAU or vehicle was given orally (dose of TAU = 4g/kg bw b.i.d.) starting the day before MPTP administration and ending on day 8. Mice were sacrificed on day 9. This study also demonstrated that TAU showed protective effects on dopaminergic neurons as indicated by an attenuated decrease in striatal DA loss due to MPTP.



**Effect of TAU on MPTP-induced decrease in striatal DA content**

| Treatment         | Striatal DA* |
|-------------------|--------------|
| Control + Control | 71.0 ± 10.6  |
| TAU + Control     | 52.0 ± 3.0   |
| Control + MPTP    | 15.9 ± 2.2   |
| TAU + MPTP        | 26.7 ± 0.9   |

\* Data are represented as ng DA/mg protein (mean ± SEM).

Example 8: Therapeutic effect of TAU in the 3-nitropropionic acid (3NP) model of Huntington's disease (HD)

HD is characterized by a progressive neuronal loss especially in the striatum. Patients with HD have a decreased activity of succinate dehydrogenase (complex II)- ubiquinol oxidoreductase (complex III) activity . Browne, *Mitochondria & Free Radicals in Neurodegenerative Diseases*, 361-380 (1997). A widely used model of HD employs an inhibitor of succinate dehydrogenase, 3-nitropropionic acid (3NP) . (Ferrante *et al.*, *Mitochondria & Free Radicals in Neurodegenerative Diseases*, 229-244, 1997). 3NP induces damage to the striatum in particular. (Brouillet *et al.*, *J Neurochem*, 60: 356-9, 1993).

Male 6-8 month old Swiss mice (National Cancer Institute; NCI, Frederick, MD) were treated with 3NP (65 mg/kg i.p.) daily for 4 days to induce mortality, neuronal cell loss and behavioral impairment with n=8/group. TAU was administered b.i.d. 4g/kg bw p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL was given to the mice one day before and every day until day 8. On day 9, the mice were perfuse fixed with 10% buffered formalin and processed for silver staining to detect neuronal damage. There was decreased mortality due to 3NP in the mice treated with TAU compared to the controls as shown below.

There was no mortality in the 3NP + TAU group, but 3 of 8 mice died in the vehicle + 3NP group.

Behavioral scoring of the 3NP treated mice was to determine whether there was any motor impairment at anytime during the study. There were 88% of the control + 3NP treated mice with behavioral impairment indicated by gross observation. A decreased incidence of impairment of only 50% was found in the TAU + 3NP treated mice.

The silver staining was analyzed by a pathologist blinded to the identity of tissue samples. There were no clear signs of neuronal damage detected in the TAU + 3NP treated mice. However, in the control + 3NP treated mice, silver staining of synaptic terminals in the striatal area (caudate/putamen area) and substantia nigra was pronounced. Silver impregnation of axons and/or synaptic terminals in the thalamus, deep mesencephalon and/or reticular formation (medulla) was also found in 80% of the control + 3NP treated mice. The substantia nigra projects to the striatum and these areas are especially vulnerable to damage by 3NP. The damage to the substantia nigra and striatum was prevented by TAU.

Example 9: Therapeutic effect of TAU in the 3-nitropropionic acid (3NP) model of epilepsy

3-nitropropionic acid (3NP) is a mitochondrial toxin that acts by inhibiting Complex II of the respiratory chain; it is used to induce brain lesions similar to those characteristic of Huntington's disease. Seizures can also be induced by the use of 3NP as a model of epilepsy and mitochondrial dysfunction. Urbanska *et al.*, Eur J Pharmacol, 359: 55-8 (1998). Male CD-1 mice (National Cancer Institute, NCI, Frederick, MD) weighing between 26-40 g were used throughout. Mice were divided into groups of 5 and animals for each group were randomly chosen from different cages to avoid possible influence of age. The mice were maintained on a 12 hr light dark cycle with free access to water and food. All experiments were performed during the light period between 9:00 and 16:00 hr. Mice (n=17-20) were given 160 mg/kg 3NP and followed for seizures. 3NP was made up at 16mg or 18mg/ml in sterile water (pH: 7.4 ). 3NP was administered i.p. in a volume of 0.1ml/10g body weight. TAU was administered 4g/kg p.o. in 0.75% hydroxypropyl-methylcellulose vehicle 2 hours prior to 3NP administration. Seizures were assessed similar to the methods previously described (Roberts &

Keith, J Pharmacol Exp Ther, 270: 505-11, 1994; Urbanska *et al.*, Eur J Pharmacol, 359: 55-8, 1998).

Behavioral observations were performed within 120 min following application of 3-NP. Three major categories of convulsive seizure response were been considered and recorded:

1. Clonic movements: the movements of the forelimbs accompanied by facial twitching;
2. Explosive clonic movements: the movement of all four limbs involving running, jumping and bouncing;
3. Tonic response: including tonic flexion and tonic extension of the all four limbs.

Mortality rate was evaluated at 120 min after 3NP injection.

3NP induced primarily clonic seizures with some mice going on to develop a running and jumping behavior that generally resulted in mortality. TAU decreased the percent incidence of clonic seizure, running seizure and mortality due to 3NP. The primary endpoint was the latency to clonic seizure. TAU increased the latency to clonic seizure from 25.0-40.8 minutes. Data are represented as mean  $\pm$  SEM.

| Endpoint                  | Control + 3NP  | TAU + 3NP      |
|---------------------------|----------------|----------------|
| % Clonic seizures         | 90.0           | 70.6           |
| % Running seizures        | 42.9           | 5.9            |
| % Mortality               | 35             | 11.8           |
| Latency to clonic seizure | 23.8 $\pm$ 0.7 | 40.8 $\pm$ 4.9 |

Example 10: Therapeutic effect of TAU in the quinolinic acid (QA) model of excitotoxicity

Quinolinic acid is an NMDA receptor agonist that has been used in models of Huntington's disease and excitotoxic damage (Beal *et al.*, J Neurosci, 11: 1649-59, 1991; Beal *et al.*, J Neurosci, 11: 147-58, 1991; Ferrante *et al.*, Exp Neurol, 119: 46-71, 1993). It can

induce severe damage to the CNS when administered directly into the striatum. The damage and/or mortality due to intrastriatal QA is likely due to a CNS etiology.

Male 6-8 month old Swiss mice (National Cancer Institute; NCI, Frederick, MD) were treated with QA (50 or 100 nmoles given bilaterally in both striatum n=8/group. TAU was administered b.i.d. 4g/kg bw p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL was given to the mice one day before and every day until day 6. On day 7 the mice were sacrificed. The QA was administered in a 2 ul volume as previously described (Tatter *et al.*, Neuroreport, 6: 1125-9, 1995).

There was a decreased mortality due to QA in the TAU treated mice. The percent of mice surviving the 7 days treated with 50 nmoles QA was 64% in the control T QA and 73% in the TAU + QA and for mice treated with 100 nmoles QA only 4% survived in the control + QA group, whereas 19% survived in the TAU + QA group. TAU demonstrated a neuroprotective effect on the excitotoxicity due to QA.

\* \* \*

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations and modifications which come within the scope of the invention as claimed.

**CLAIMS**

1. A method for treating or preventing pathophysiological consequences of mitochondrial respiratory chain dysfunction in a mammal comprising administering to said mammal in need of such treatment or prevention an effective amount of a pyrimidine nucleotide.
2. A method as in claim 1 wherein said respiratory chain dysfunction is caused by a mutation, deletion, or rearrangement of mitochondrial DNA.
3. A method as in claim 1 wherein said respiratory chain dysfunction is caused by defective nuclear-encoded protein components of the mitochondrial respiratory chain.
4. A method as in claim 1 wherein said respiratory chain dysfunction is caused by aging.
5. A method as in claim 1 wherein said respiratory chain dysfunction is caused by administration of cytotoxic cancer chemotherapy agents to said mammal.
6. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex I activity.
7. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex II activity.
8. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex III activity.
9. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex IV activity.
10. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex V activity.

11. A method as in claim 1 wherein said pyrimidine nucleotide precursor is selected from the group consisting of uridine, cytidine, an acyl derivative of uridine, an acyl derivative of cytidine, orotic acid, an alcohol ester of orotic acid, or a pharmaceutically acceptable salt thereof.
12. A method as in claim 11 wherein said pyrimidine nucleotide precursor is an acyl derivative of cytidine.
13. A method as in claim 11 wherein said pyrimidine nucleotide precursor is an acyl derivative of uridine.
14. A method as in claim 11 wherein said acyl derivative of uridine is 2',3',5'-tri-O-acetyluridine.
15. A method as in claim 11 wherein said acyl derivative of uridine is 2',3',5'-tri-O-pyruvyluridine.
16. A method as in claim 11 wherein the alcohol substituent of said alcohol ester of orotic acid is ethanol.
17. A method as in claim 11 wherein said pyrimidine nucleotide precursor is cytidine diphosphocholine.
18. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered orally.
19. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered in a dose of 10 to 1000 milligrams per kilogram of bodyweight per day.
20. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered in a dose of 100 to 300 milligrams per kilogram of bodyweight per day.

21. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a congenital mitochondrial disease.
22. A method as in claim 21 wherein said congenital mitochondrial disease is selected from the group consisting of MELAS, LHON, MERRF, MNGIE, NARP, PEO, Leigh's Disease, and Kearns-Sayres Syndrome.
23. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a neurodegenerative disease.
24. A method as in claim 23 wherein said neurodegenerative disorder is Alzheimer's Disease.
25. A method as in claim 23 wherein said neurodegenerative disorder is Parkinson's disease.
26. A method as in claim 23 wherein said neurodegenerative disorder is Huntington's Disease.
27. A method as in claim 23 wherein said neurodegenerative disorder is age-related decline in cognitive function.
28. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a neuromuscular degenerative disease.
29. A method as in claim 28 wherein said neuromuscular degenerative disease is selected from the group consisting of muscular dystrophy, myotonic dystrophy, chronic fatigue syndrome, and Friedreich's Ataxia.
30. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is developmental delay in cognitive, motor, language, executive function, or social skills.
31. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is selected from the group consisting of epilepsy, peripheral -

neuropathy, optic neuropathy, autonomic neuropathy, neurogenic bowel dysfunction, sensorineural deafness, neurogenic bladder dysfunction, migraine, and ataxia.

32. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is selected from the group consisting of renal tubular acidosis, dilating cardiomyopathy, steatohepatitis, hepatic failure, and lactic acidemia.
33. A method for preventing death or functional decline of post-mitotic cells in a mammal due to mitochondrial respiratory chain dysfunction comprising administration of an effective amount of a pyrimidine nucleotide precursor.
34. A method as in claim 33 wherein said post-mitotic cells are neurons.
35. A method as in claim 33 wherein said post-mitotic cells are skeletal muscle cells.
36. A method as in claim 33 wherein said post-mitotic cells are cardiomyocytes.
37. A method for treating developmental delay in cognitive, motor, language, executive function, or social skills in a mammal comprising administration of an effective amount of a pyrimidine nucleotide.
38. A method as in claim 37 wherein said developmental delay is pervasive developmental delay or PDD-NOS.
39. A method as in claim 37 wherein said developmental delay is Attention Deficit/Hyperactivity Disorder.
40. A method as in claim 37 wherein said developmental delay is Rett's Syndrome.
41. A method as in claim 37 wherein said developmental delay is autism.



42. A method for reducing side effects of cytotoxic cancer chemotherapy agents by administering a pyrimidine nucleotide precursor, where said cytotoxic chemotherapy agent is not a pyrimidine nucleoside analog.
43. A method as in claim 42 wherein said side effects of cytotoxic cancer chemotherapy are selected from the group consisting of peripheral neuropathy, chemotherapy-induced menopause, chemotherapy-associated fatigue, and depressed appetite.
44. A method for diagnosing mitochondrial disease by administering a pyrimidine nucleotide precursor and assessing clinical improvement in signs and symptoms in a mammal.
45. A compound selected from the group consisting of 2',3',5'-tri-O-pyruvyluridine, 2',3'-di-O-pyruvyluridine, 2',5'-di-O-pyruvyluridine, 3',5'-di-O-pyruvyluridine, 2'-O-pyruvyluridine, 3'-O-pyruvyluridine, and 5'-O-pyruvyluridine.
46. A pharmaceutical composition comprising:
- (a) a pyrimidine nucleotide precursor or a pharmaceutically acceptable salt thereof, and
  - (b) pyruvic acid, a pharmaceutically acceptable salt thereof, or a pyruvic acid ester.
47. A method as in Claim 1 further comprising administering pyruvic acid, a pharmaceutically acceptable salt thereof, or a pyruvic acid ester.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/19725

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01N 43/04; A61K 31/70  
US CL : 514/49

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/49

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: medline, caplus, biosis, embase, biotechds

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category*             | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.                             |
|-----------------------|--|---|
| X<br>—<br>A           | US 5,470,838 A (VAN BORSTEL) 28 November 1995, col. 13, lines 54-56; and col. 15, lines 6-12.  | 1, 11-14<br>—<br>15,16,44                         |
| X<br>—<br>Y<br>—<br>A | SECADES et al. CDP-Choline: Pharmacological and Clinical Review. Methods and Findings in Experimental and Clinical Pharmacology. October 1995, Vol. 17, Supplement 1, pages 1-54, especially abstract, page 4, last full paragraph through page 5 col. 1; page 21, col.2 through page 22, and Figs. 11-13; page 38, col. 2 through page 39 col. 1; pages 40-43, especially page 41, col.1, and Table 18. | 1, 11, 17<br>—<br>1-11,18-42, 46<br>—<br>15,16,44 |



Further documents are listed in the continuation of Box C.



See patent family annex.

|   |  |
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| *O* document referring to an oral disclosure, use, exhibition or other means  |  |
| *P* document published prior to the international filing date but later than the priority date claimed  |  |

Date of the actual completion of the international search

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/19725

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*    | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.   |
|--------------|--|-------------------------|
| X<br>--<br>Y | Database Medline on STN AN 94049698, KEILBAUGH et al. Anti-human immunodeficiency virus type 1 therapy and peripheral neuropathy: prevention of 2', 3'-dideoxycytidine toxicity in PC 12 cells, a neuronal model, by uridine and pyruvate. Molecular Pharmacology. October 1993. Vol. 44. No. 4. pages 702-706, abstract only. | 45<br>----<br>43        |
| Y            | DYKENS, J.A. Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca <sup>2+</sup> and Na <sup>+</sup> : Implications for neurodegeneration. Journal of Neurochemistry. 1994, Vol. 63, pages 584-591, especially lines 24-28 of abstract; and page 589, col. 1. first paragraph.       | 1-11,18-42, 46          |
| Y            | BODNAR et al. Respiratory-deficient human fibroblasts exhibiting defective mitochondrial DNA replication. Biochem. J. 1995, Vol. 305, pages 817-822, especially abstract lines 6-10.   | 2-10, 20, 21, 25-42, 46 |